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Response of the mouse sublingual gland to spaceflight

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Abstract

The ultrastructure and immunohistochemistry of secretory proteins of sublingual glands were studied in mice flown on the US space shuttles Discovery (Space Transportation System [STS]-131) and Atlantis (STS-135). No differences in mucous acinar or serous demilune cell structure were observed between sublingual glands of ground control and flight mice. In contrast, previous studies showed autophagy and apoptosis of parotid serous acinar cells in flight mice. The expression of parotid secretory protein (PSP) in sublingual demilune cells of STS-131 flight mice was significantly increased compared to ground control mice but decreased in STS-135 flight mice. Similarly, mucin (Muc19) expression in acinar cells and PKA-RII expression in demilune cells were increased in STS-131 flight mice and decreased in STS-135 flight mice, but not significantly. Demilune cell and parotid protein (DCPP) was slightly decreased in mice from both flights, and nuclear PKA-RII was slightly increased. These results indicate that the response of salivary glands to spaceflight conditions varies among the different glands, cell types and secretory proteins. Additionally, the spaceflight environment, including the effects of microgravity, modifies protein expression. Determining changes in

salivary proteins may lead to development of non-invasive methods to assess the physiological status of astronauts.

Keywords: microgravity, salivary gland structure, protein expression, electron microscopy, immunohistochemistry

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Human history in space began in the 1960s and has expanded since then. Advancements in space technology have made it possible to answer some of the questions about distant planets and have generated interest in possible human missions to explore these worlds. To carry out these missions, however, requires a more complete understanding the effects of spaceflight and microgravity on biological processes. There are a number of ways to obtain microgravity-associated data including actual spaceflight missions and ground-based studies that simulate weightlessness (bed rest models, hindlimb suspension models, clinostats). However, spaceflight is a unique environment which cannot be totally duplicated.

Rodents are the most used animal model in space research, although primates as well as cold-blooded vertebrates and invertebrates also have been flown in space (1). Rodents have advantages over other animals due to their small size, structural and functional similarities to humans, ease of maintenance, and less expense.

Previous studies revealed that bone loss, decreased immune system function, alterations in gastrointestinal microbial flora and cardiovascular deconditioning are major consequences of weightlessness on biological systems (2–5). Spaceflight also affects oral hard and soft tissues. Limited information is available about the effects of microgravity on oral tissues including the mandible and teeth (6–9), salivary glands (10–13) and saliva (14).

Salivary glands and their secretions can be used to study environmentally-induced changes in cellular and molecular processes by measuring secretory proteins that are specific cell type markers or are indicators of specific cell functions. Saliva from the major and minor salivary glands contains proteins that can serve as indicators of an organism's systemic physiology and pathology (15). Our previous studies of the rodent parotid gland showed specific structural and molecular alterations following spaceflight (10–13). The parotid gland is a pure serous gland, secreting a variety of proteins and glycoproteins with diverse functions. In contrast, the sublingual gland consists predominantly of mucous cells, with a few serous demilune cells. The main product of the sublingual gland is mucin, with a smaller contribution of proteins and glycoproteins from the demilune cells.

Based on the working hypothesis that microgravity modifies ultrastructure and protein expression, it should be possible to measure such changes in the sublingual glands of mice flown on two US space shuttle missions. This study was designed to determine structural changes in the mouse sublingual gland using transmission electron microscopy (TEM), to determine cellular distribution and expression of selected sublingual gland proteins by immunohistochemistry (IHC), and to compare these changes to those observed in the parotid gland.

Material and Methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee at the National Aeronautics and Space Administration (NASA) and conformed to the U.S. National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals* (16). The samples were made available through the NASA Biospecimen Sharing Program.

Eight adult C57BL/6 female mice, 16-23 wk old, were flown for 15 d on space shuttle Discovery (Space Transportation System [STS]-131) in Animal Enclosure Modules (AEMs), specially designed cages that provide ventilation, lighting, waste collection, food and water for the rodents used in spaceflight research (17). Eight ground control mice were also housed in AEMs for the same length of time as the flight. Samples also were collected from 7 C57BL/6 female mice, 9 wk old, flown on space shuttle Atlantis (STS-135) for 13 d, along with 4 ground control mice housed in AEMs for the same length of time. All mice were provided with food (NASA rodent food bars) (18) and water *ad libitum*.

Tissue dissections of the flight mice began approximately 3 h after landing; samples were collected from the AEM ground control mice 48 h after the flight mice. The mice were anesthetized with isoflurane and killed by cardiac puncture and exsanguination. Sublingual salivary glands were prepared for morphological and IHC studies. Routine morphology samples were placed in 2.5% glutaraldehyde/2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4; samples for IHC were fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer. After fixation, samples for morphological analyses were rinsed several times and stored at 4°C in 0.1 M cacodylate buffer, pH 7.4; samples for IHC were stored in 1% paraformaldehyde in 0.1 M cacodylate buffer. All samples were shipped cold by express service from Kennedy Space Center to UConn Health.

Samples for EM morphological study were postfixed in OsO_4 and processed by standard methods (19) for embedding in PolyBed epoxy resin (Polysciences, Warrington, PA, USA). Thin sections were collected on copper grids, stained with uranyl acetate and lead citrate, and examined and photographed in a Hitachi H7650 TEM equipped with an AMT digital camera.

For IHC, samples were embedded in paraffin, 5-µm sections were collected on Superfrost Plus slides (Fisher Scientific, Waltham, MA, USA) and stained as described previously (20) using the Vectastain Elite kit for rabbit IgG (Vector Laboratories, Burlingame, CA, USA). Primary antibodies used were to sublingual gland mucin (Muc19), a product of the mucous acinar cells, parotid secretory protein (PSP), an antibacterial protein of the serous demilune cells, demilune cell and parotid protein (DCPP), and the type II regulatory subunit of protein kinase A (PKA-RII), an intracellular regulatory protein and stress marker (Table 1). Controls included omission of the primary antibody, and substitution of pre-immune or non-immune serum or immunoglobulin for the primary antibody. The labeled sections were photographed with a Leitz Orthoplan microscope equipped with a Nikon Coolpix 5000 camera.

Table 1.

Antibodies Used for Immunohistochemistry.

Antibody	LM Dilution	EM Dilution	Reference
Muc19	1:200 – 1:400	1:10 – 1:30	(21)
PSP	1:3000 – 1:6000	1:150 – 1:400	(22)
DCPP	1:3000 – 1:6000	1:150	(12,13)
PKA-RII	1:500 – 1:2000	1:80 – 1:100	(23,24)

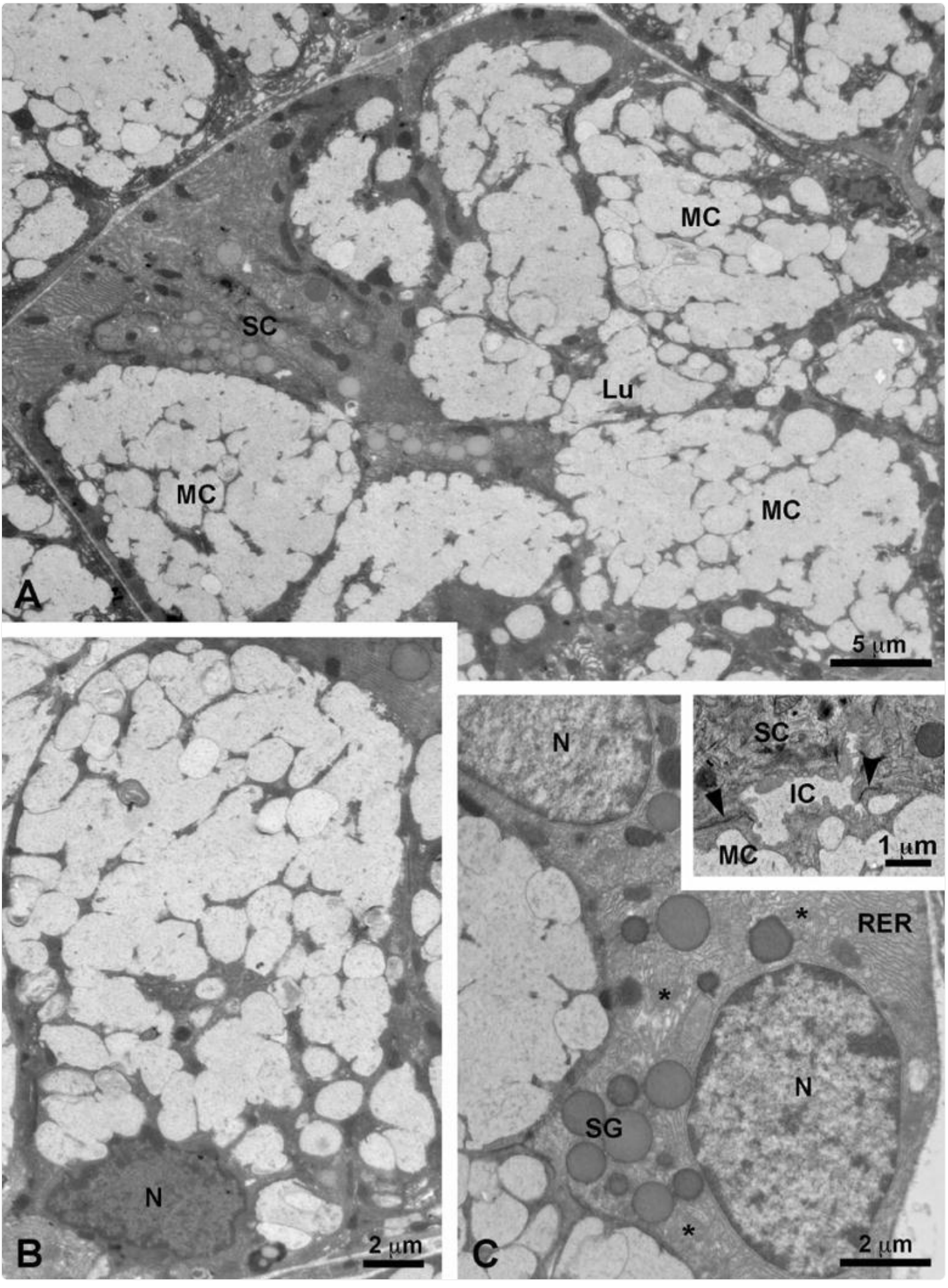
Portions of the samples fixed for IHC were prepared for electron microscopic immunogold labeling. OsO_4 postfixation was omitted, the samples were dehydrated in cold methanol solutions, embedded in LR Gold resin (Electron Microscopy Sciences, Hatfield, PA, USA) and polymerized in UV light at −20°C. Thin sections from each mouse of the ground control and flight groups from each mission were collected on formvar-coated nickel grids. Non-specific binding was blocked with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 5% normal goat serum (NGS), and the sections were incubated overnight at 4°C with primary antibodies (Table 1) diluted in blocking solution. After rinsing with PBS, the grids were incubated with goat anti-rabbit IgG labeled with 15 nm gold particles (Amersham, Little Chalfont, Bucks, UK, or Aurion, Electron Microscopy Sciences, Wageningen, The Netherlands), diluted in 1% BSA–PBS. The grids were washed with PBS and distilled water and stained with uranyl acetate and lead citrate.

Electron micrographs of the labeled sections were obtained in an unbiased manner by selecting cells at low magnification so that gold particles were not visible, then photographing the selected field at 10,000×. Photographs of at least 10 fields each of mucous secretory droplets and serous secretory granules, 5-10 fields each of mucous and serous cell nuclei, and 5 fields of empty plastic were photographed in one section from each mouse. Quantitative analyses of the immunogold labeled micrographs were performed in Photoshop (CS2 v. 9.0.2 or CS4 Extended v. 11.0.2, Adobe Systems, San Jose, CA, USA) using a grid overlay method as described previously (13). Labeling densities for each organelle for each mouse were calculated as mean number of gold particles/μm^2. For direct comparison between different immunogold labeling experiments, the labeling densities were converted to percentages of the corresponding ground control means. Differences in expression of each protein between the flight and ground control groups were determined using the t-test function in Microsoft Excel or StatPlus (v.6.1.60, AnalystSoft, Walnut, CA, USA).

Results

Morphology and IHC reactivity in the sublingual salivary glands of flight animals was determined, quantified and compared with those of ground control animals. In thin sections examined in the TEM, the secretory end pieces, or acini, were composed of mucous cells arranged around the lumen, and serous cells interposed between adjacent mucous cells or forming a demilune of two or three cells (Fig. 1A). The characteristic feature of mucous acinar cells was the presence of electron lucent secretory droplets filling the supranuclear cytoplasm (Fig. 1B). An extensive Golgi complex and rough endoplasmic reticulum were located mainly in the basolateral cytoplasm, along with mitochondria and occasional lysosomes. The nucleus was relatively electron dense and located close to the base of the cell. Serous cells typically contained a few to several moderately electron dense secretory granules in the apical cytoplasm, a well-developed Golgi complex, and abundant rough endoplasmic reticulum (Fig. 1C). The nucleus was generally spherical and less dense than the nucleus of mucous cells. An intercellular canaliculus (inset, Fig. 1C) connected the luminal surface of the demilune cells to the main lumen of the mucous acinus. Short intercalated ducts (Fig. 2) consisting of cuboidal cells connected the acini to the striated ducts. Occasionally, a few intercalated duct cells close to the acinus had accumulations of small mucous-like secretory droplets (inset, Fig. 2). The striated ducts consisted of columnar epithelial cells with abundant mitochondria and extensive infoldings of the basal cell membrane. There were no significant differences in the ultrastructure of sublingual mucous cells, serous cells or duct cells of the animals on either flight when compared to those of the corresponding ground control animals.

Figure 1.


A
MC
SC
Lu
MC
MC
5 µm
B
N
2 µm
C
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SC
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MC
1 µm
*
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N
SG
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2 µm

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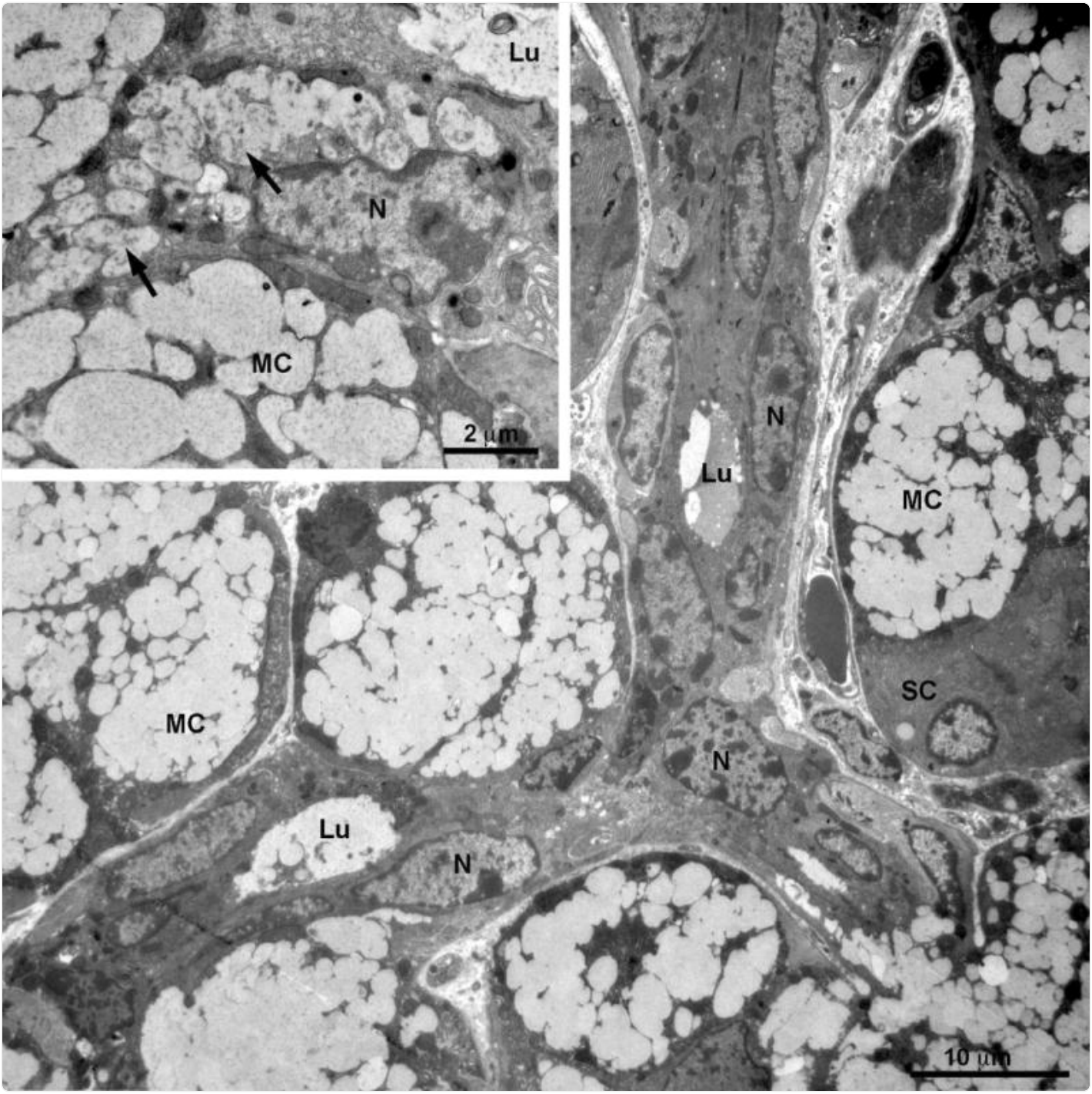
A, STS-135 ground control. Electron micrograph of sublingual gland acinus. Several mucous cells (MC) containing pale mucous secretory droplets are present around the lumen (Lu). Portions of three serous cells (SC) are located between the mucous cells. **B**, STS-135 flight. Higher magnification of a mucous acinar cell. A dense nucleus (N) is located basally, and the supranuclear cytoplasm is filled with mucous droplets, many of which have fused together. **C**, STS-135 flight. Higher magnification of parts of two serous cells. Abundant rough endoplasmic reticulum (RER) is present in the basal cytoplasm. Dense secretory granules (SG) and portions of the Golgi complex (*) can be seen. The nuclei (N) are less dense than those of the mucous cells. Inset: STS-135 ground control. An intercellular canaliculus (IC), delimited by junctional complexes (arrowheads), is present between a serous cell and a mucous cell.

Figure 2.



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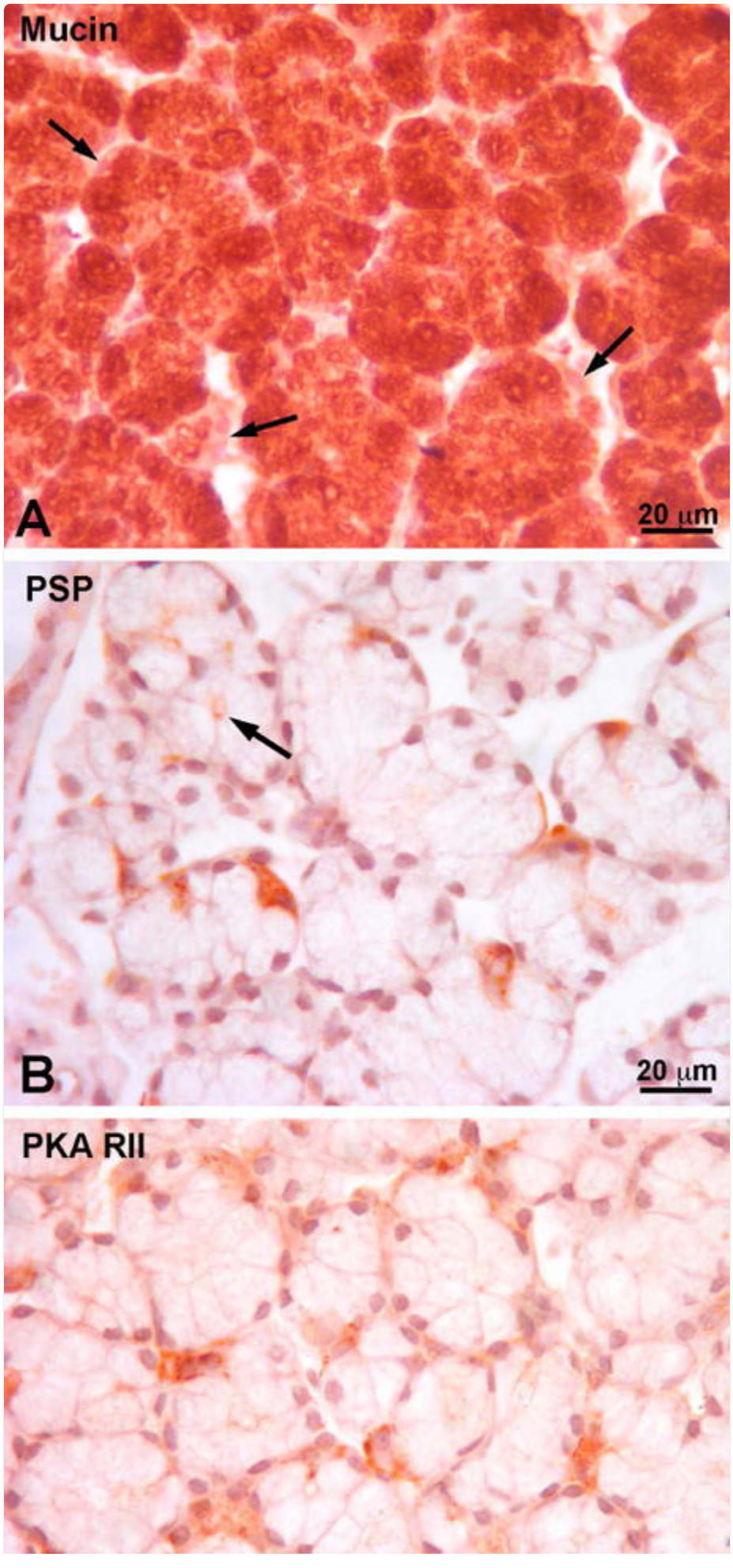
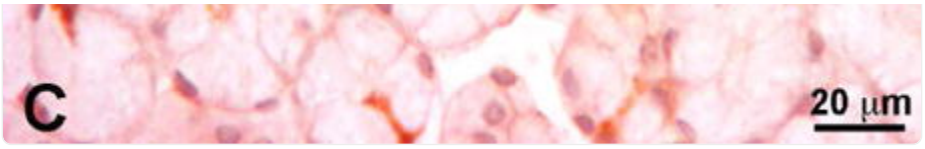
STS-135 ground control. A branching intercalated duct seen mainly in longitudinal section lies between several acini. Intercalated duct lumen (Lu); intercalated duct cell nuclei (N). Inset: STS-131 flight. An intercalated duct cell adjacent to mucous acinar cells contains mucous-like secretory droplets (arrows) of

smaller size and different appearance than those of the acinar cells.

IHC for mucin (Muc19) showed intense reactivity in the mucous acinar cells (Fig. 3A). In contrast, PSP (Fig. 3B), DCPP (not shown) and PKA-RII (Fig. 3C) were present in serous demilune cells. No differences in reactivity between flight and ground control mice could be detected at the light microscopic level.

Figure 3.


Mucin
A
20 μm
PSP
B
20 μm
PKA RII



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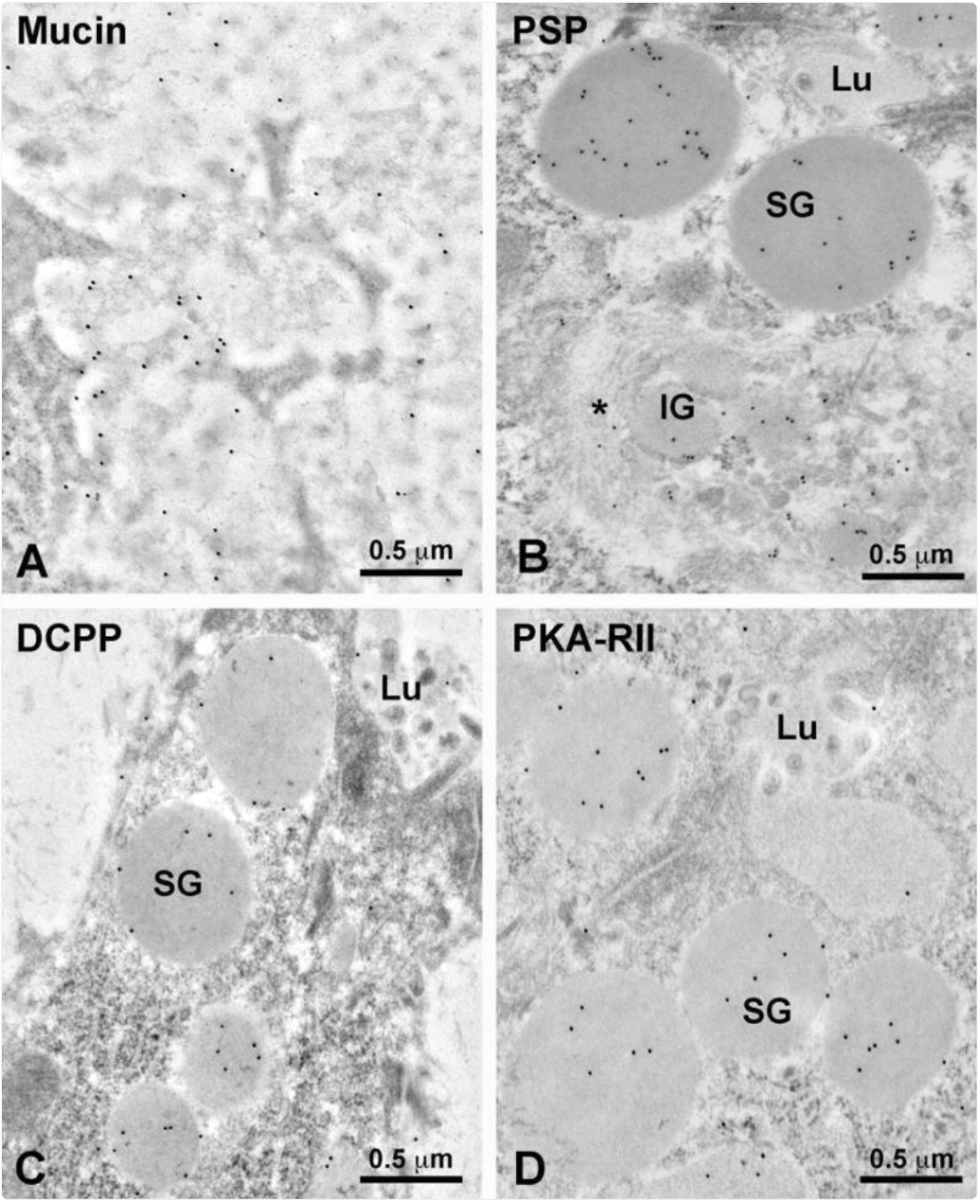
A, STS-131 ground control. IHC localization of mucin (Muc19) in sublingual mucous acinar cells. Serous cells (arrows) are unreactive. **B**, STS-131 ground control. Localization of PSP in serous cells. Some reactivity is present in the lumina of mucous acini (arrow). **C**, STS-131 flight. Localization of PKA-RII in serous cells.

Immunogold labeling for mucin (Muc19) revealed reactivity in the droplets of the mucous acinar cells (Fig. 4A). The labeling density for mucin was slightly greater in glands of STS-131 flight mice and slightly less in STS-135 flight mice than in the corresponding ground controls, although the differences were not significant (Fig. 5).

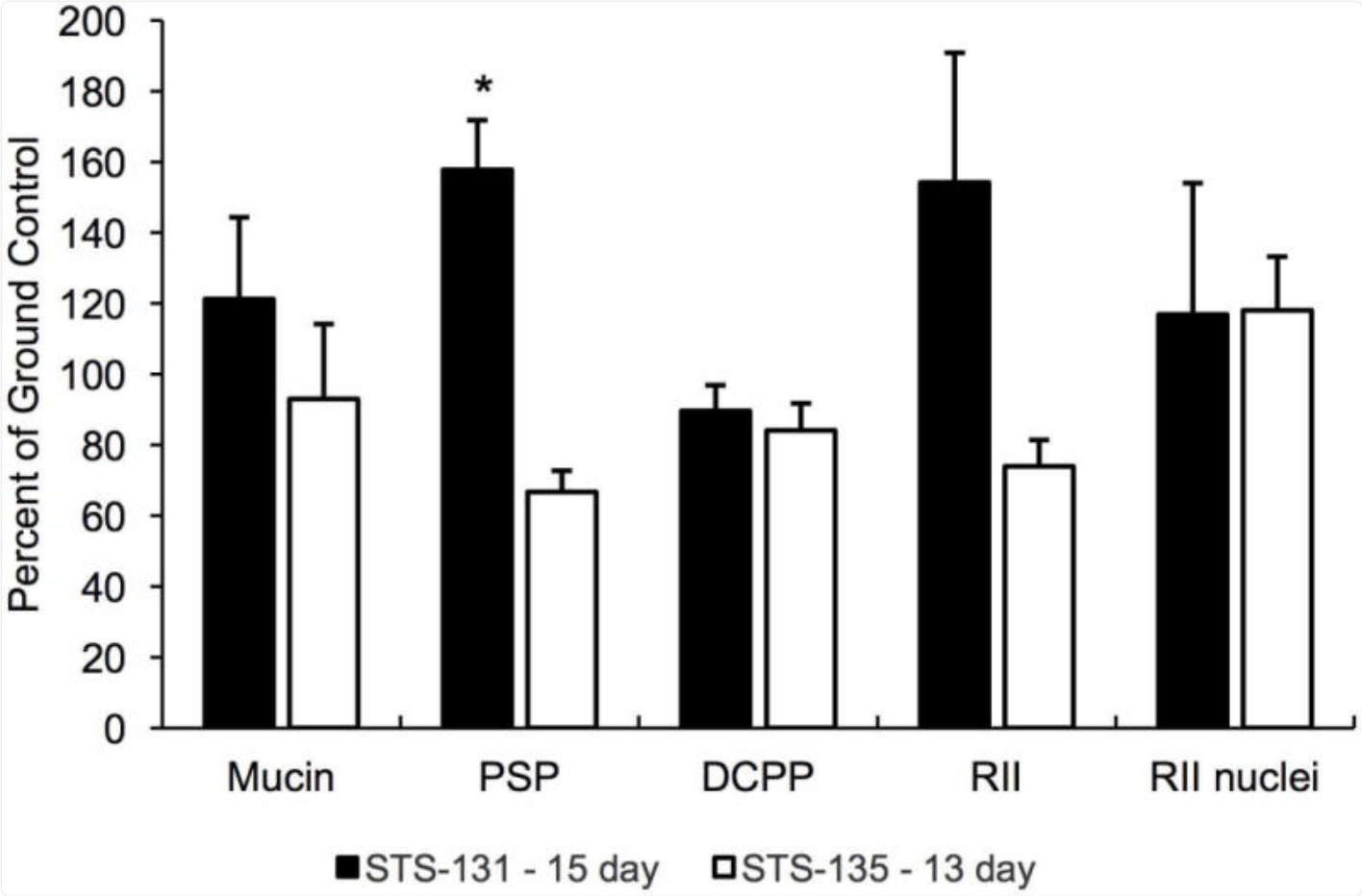
Figure 4.


Mucin
A
0.5 μm
PSP
Lu
SG
*
IG
B
0.5 μm
DCPP
Lu
SG
C
0.5 μm
PKA-RII
Lu
SG
D
0.5 μm

Open in a new tab

A, STS-131 flight. Immunogold labeling of sublingual mucous acinar cell with anti-mucin antibody. Gold particles are present over the pale mucous droplets. **B**, STS-135 ground control. Labeling of serous demilune cell with anti-PSP antibody. Gold particles are located over the dense serous granules (SG) and immature granules in the Golgi region (IG). A few particles are present over the Golgi saccules (*). Lumen (Lu). **C**, STS-131 ground control. Labeling of serous demilune cell with anti-DCPP antibody. Gold particles are present over the serous granules. **D**, STS-135 ground control. Labeling of serous demilune cell with anti-PKA-RII antibody. Gold particles are present over the serous granules.

Figure 5.



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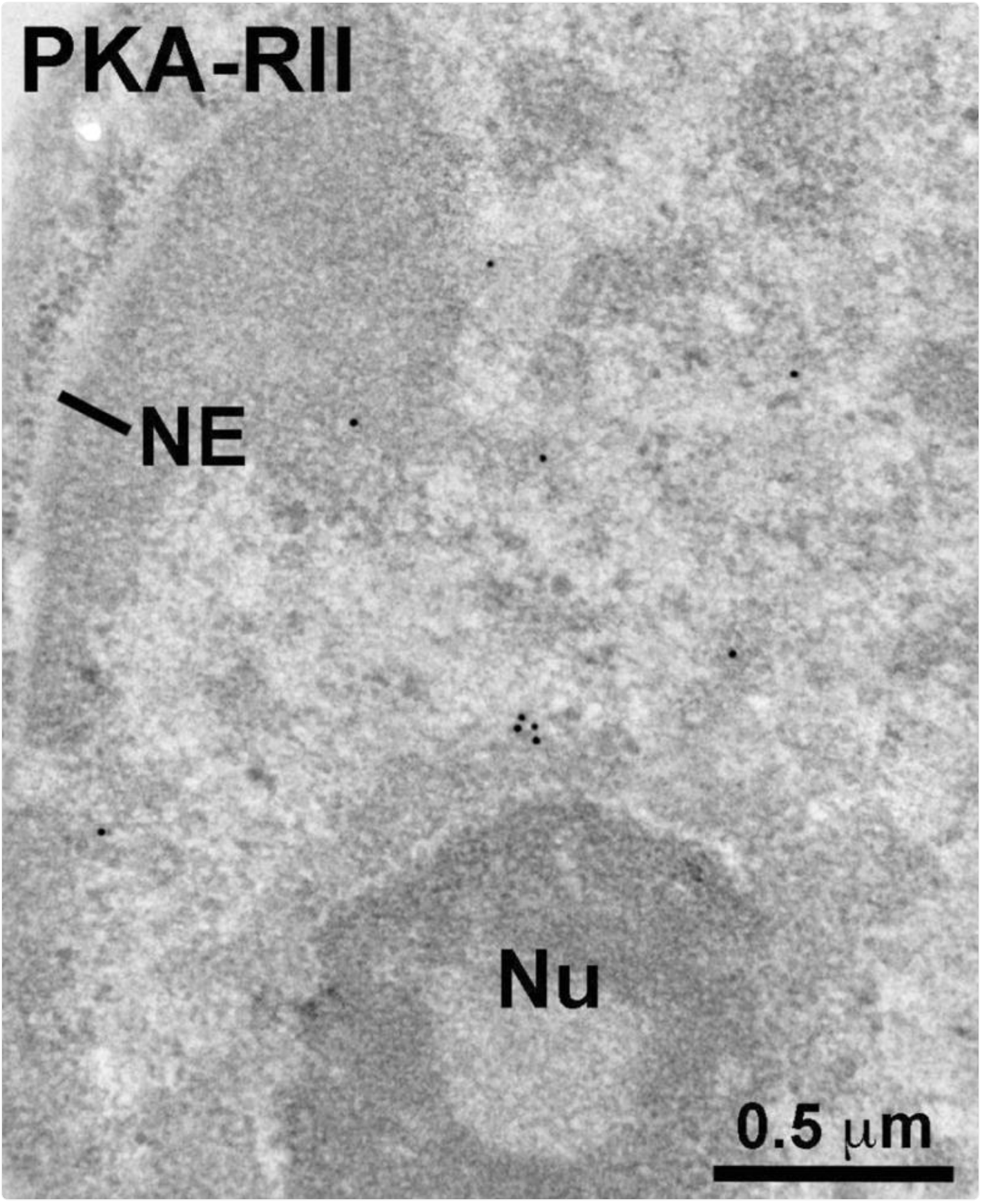
Quantitative analysis of immunogold labeling for mucin in the mucous acinar cell secretory droplets, PSP, DCPP and PKA-RII in the serous demilune cell secretory granules, and PKA-RII in the serous cell nuclei of the sublingual glands of flight mice from STS-131 and STS-135. Results are expressed as a percentage (± SEM) of the AEM-housed ground control mice. *, $p<0.05$ compared to control.

Immunogold labeling for PSP was present in the granules of serous cells (Fig 4B). PSP expression was significantly increased ($p<0.05$) in the glands of STS-131 flight mice compared to ground control mice (Fig. 5). In contrast, PSP expression appeared decreased in the glands of STS-135 flight mice, although not significantly due to substantial variation in labeling among the ground control mice. However, the difference in PSP labeling densities between the two flight groups was highly significant ($p<0.001$).

Immunogold labeling for DCPP also was present in the granules of serous cells (Fig. 4C). Compared to the corresponding ground control mice, DCPP labeling was slightly, but not significantly, decreased in the glands of mice on both flights (Fig. 5).

Immunogold labeling showed that PKA-RII was present in the granules of serous cells (Fig. 4D). The differences between flight and control mice were not significant, however PKA-RII labeling density was increased in the glands of STS-131 mice but decreased in the glands of STS-135 mice (Fig. 5). The difference in labeling densities between the two groups of flight mice showed a trend toward significance ($p=0.063$). PKA-RII also was present at low levels in the nuclei of the demilune cells (Fig. 6). Labeling of the nuclei of mice on both flights was slightly increased (Fig. 5), but not significantly.

Figure 6.



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STS-135 ground control. Immunogold labeling of the nucleus of a serous demilune cell with anti-PKA-RII antibody. Nuclear envelope (NE); nucleolus (Nu).

Discussion

Studies to determine whether microgravity affects the morphology of salivary gland secretory and duct cells indicate that no significant changes occurred in the sublngual gland. The parotid glands of the same mice on both flights showed an increase in autophagic vacuoles in the serous acinar cells, an increased incidence of apoptotic acinar cells, and the presence of large endocytic vacuoles containing acinar secretory proteins in intercalated and striated duct cells (12,13). This likely represents differences in the genetic makeup, and metabolic and secretory functions of the two glands.

To determine if microgravity affects the expression of sublingual gland secretory proteins, cell type markers or indicators of specific cell functions with known cellular or oral function were selected. Mucins function in oral health maintenance by protecting oral tissues, providing lubrication and antibacterial activity, and forming a barrier to mechanical and chemical insults (25,26). Muc19 is the major product of mouse sublingual mucous acinar cells (27); its expression increases during perinatal development of the sublingual gland (28). The results suggest that microgravity and the spaceflight environment have little or no effect on sublingual gland mucin expression.

PSP, a major product of rodent parotid gland acinar cells (29,30), is present at lower levels in rodent sublingual gland serous demilune cells and is transiently present in the developing submandibular gland (22,31). Human genome sequence analysis has revealed that the human homologue of rodent PSP, a member of the PLUNC (palate, lung, nasal clone) family of secretory proteins, is expressed in human parotid as well as submandibular glands (32). Mouse and human PSP have been shown to bind to and agglutinate several types of oral and digestive tract bacteria (33,34). PSP expression was significantly increased in the sublingual glands of flight mice from STS-131, but was decreased, although not significantly, in flight mice from STS-135.

The difference in ages of the mice on the two flights (16-23 wk at launch for STS-131 vs. 9 wk for STS-135) may be related to the different responses and in particular to the significant difference in PSP labeling densities between the two flights. Age-related changes in sublingual gland PSP expression have been shown previously (30,35). In the parotid, PSP expression in these mice did not differ from controls (12,13), suggesting that regulation of this protein may differ in the two glands.

Demilune cell and parotid protein (DCPP) is found mainly in serous demilune cells of mouse sublingual gland and

intercalated ducts of parotid glands (36). The structural similarities between the gene sequences of mouse DCPP and its human homologue, HRPE 773, suggest that DCPP may have antibacterial function in the oral cavity (37). Immunogold labeling showed no significant differences in sublingual gland DCPP expression compared to controls for mice on either flight. DCPP expression was significantly increased in the parotid glands of STS-131 flight animals (12) but was not different from controls in STS-135 flight animals (13). This suggests that, as for PSP, the regulation of DCPP expression differs in these two glands.

Protein phosphorylation by protein kinase A (PKA) affects many cellular processes, including exocytosis of stored secretory proteins by salivary gland cells (38,39). The regulatory subunits of type II PKA (RII) are polyfunctional proteins that themselves are involved in various cell functions including growth, development and gene expression. Previous studies have shown that RII is present in the secretory granules of parotid acinar cells in rats and mice (12,40), in the secretory granules of human parotid and submandibular serous acinar cells and sublingual demilune cells (41) and is released into saliva (42).

The secretory granules of the demilune cells of STS-131 mice exhibited a trend toward a greater RII labeling density than those of STS-135 mice, but the labeling density of the granules of the flight mice was not statistically different from the ground controls. The increased RII in the sublingual gland of STS-131 differs from the results for secretory granules of the parotid glands for the mice on these two flights and on the 30-d Bion-M1 mission. In all three missions, labeling of parotid acinar cell granules of flight animals was slightly, but not significantly, lower than that of the ground control animals (12,13). RII shows a large, transient increase upon acute stress, but a steady decrease upon continued, chronic stress and a trend toward return to baseline after 4 or 5 d (43,44). A decrease during flight therefore might have been obscured by the stress of landing.

PKA has an important role in the regulation of gene expression (45,46). RII is present in the nuclei of parotid gland acinar cells (40,47), and the amount of nuclear RII varies in response to changes in physiological status (23,48). RII is present in the nuclei of sublingual demilune cells, but the labeling density of the nuclei was only slightly, and not significantly, greater in flight mice than in controls. Spaceflight, therefore, has relatively little effect on sublingual gland genes regulated by the cyclic AMP-PKA system.

Protein expression differences without morphological changes in salivary glands can indicate adaptation to a changed environment. Specific differences in sublingual gland protein expression were found between the 15-d STS-131 flight and the 13-d STS-135 flight. The expression of PSP, PKA-RII, and mucin was greater in the glands of mice from STS-131 than in those from STS-135. It is unlikely that the two-day difference in the length of the flights could account for these observed differences, although GHOSH *et al.* (8) showed a difference in the response of mandibular bone to microgravity between the STS-131 and STS-135 flight mice. The difference in age of the mice on the two flights, however, is a significant factor. The results also highlight the difference in response of the parotid and sublingual glands to spaceflight. Future studies comparing samples from different flight durations and larger numbers of animals would

address these questions. Samples collected during different time frames after landing will help determine if and when the values return to preflight levels. Long lasting alterations may have irreversible physiologic effects.

Determining the effects of spaceflight on biological systems will be useful for developing countermeasures as well as providing insight into the unknowns of this unique environment. Gravitational changes, temperature fluctuations, vibration, noise and circadian and sleep disturbances are some of the parameters contributing to astronauts' physical and psychological stress level and resulting in hormonal changes (5). Changes in salivary secretory proteins are reflected in saliva. Easy access to, and non-invasive collection of saliva makes it a convenient medium to measure physiological and pathological changes in humans. Salivary tests have become a more commonly used diagnostic tool in recent years (15,49–51). Data from spaceflight research will be important in developing salivary test kits that can be used to measure human responses to spaceflight.

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Footnotes

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Conflicts of Interest

The authors declare no potential conflicts of interest, with respect to the research, authorship or publication of this article.

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